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EFFECTS OF LYMECYCLINE ON *MYCOPLASMA PULMONIS*-INDUCED ARTHRITIS IN MICE

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Summary.—The effect of lymecycline treatment on arthritis in C3H mice produced 5 months previously by i.v. inoculation of *Mycoplasma pulmonis* was examined. Treatment had little effect on the severity of the clinical disease. However, there was a marked reduction the severity of the histopathological inflammatory reaction in joints from lymecycline-treated mice when compared with untreated controls. This reduction was associated with eradication of viable mycoplasmas from the joints. The findings suggest that persistent arthritis in C3H mice is due to the continued presence of viable *M. pulmonis* organisms in the joint tissues.

MYCOPLASMAS have been considered as a possible cause of rheumatoid arthritis largely because of their association with arthritis in a number of other animal species (see Taylor-Robinson and Taylor, 1976). Further, some of these arthritic diseases persist for long periods of time and have histological features in common with rheumatoid arthritis. These observations have stimulated an interest in the pathogenesis of mycoplasma-induced chronic arthritis. It has been suggested that the persistent nature of some of these diseases may be due to the continued presence of viable mycoplasmas or non-viable mycoplasma antigen in the joint (Cole, Golightly-Rowland and Ward, 1975; Ennis, Johnson and Decker, 1972), or the result of an auto-immune reaction (Harwick *et al.*, 1973*b*). If persistence of viable mycoplasmas is important in the development of chronic arthritis, it should be possible to recover organisms from the joints at all stages of the disease. Although mycoplasmas can be isolated from affected joints only in the early phase of some chronic arthritic diseases—*e.g. M. hyorhinis* in pigs (Barden and Decker, 1971), and *M. pulmonis* and *M. arthritidis* in rabbits (Cole *et al.*, 1977)—it is possible to recover mycoplasmas from arthritic joints at all stages of the disease in other infections—*e.g. M. pulmonis* in mice (Cole *et al.*, 1975). However, there are discrepancies in the reported isolation rates of *M. pulmonis* from arthritic joints of mice. Thus, whereas some workers isolated *M. pulmonis* from nearly all arthritic joints (Cole *et al.*, 1975; Taylor and Taylor-Robinson, unpublished observations), others reported the isolation of this mycoplasma from only a small percentage of arthritic joints, regardless of the duration of the disease (Harwick *et al.*, 1973*a*). The latter observation led to the suggestion that arthritic joints from which mycoplasmas were not recovered may have been affected by some immunological mechanism secondary to the infection (Harwick *et al.*, 1973*a*, *b*).

Another approach to determine whether persistence of viable mycoplasmas is responsible for chronic arthritis is to treat animals with anti-mycoplasma drugs and examine the effects of such treatment on the disease and on recovery of organisms from joint tissues. Gold salts are effective in preventing mycoplasma-induced arth-

ritis in rats and mice when given at the time of infection (Findlay, Mackenzie and MacCallum, 1940; Hannan, 1977). However, it has been suggested that the beneficial effect of gold salts is largely the result of their action on the host's inflammatory and/or immune response (Hannan, 1977). The effect of tetracyclines on the course of mycoplasma-induced arthritis in rats and mice has also been examined (Hannan, 1977; Harwick, Kalmanson and Guze, 1974). Whereas tetracycline treatment started at the time of inoculation of mice with *M. pulmonis* was effective in preventing the arthritis (Hannan, 1977), treatment delayed for several weeks after inoculation had little effect either on the severity of clinical disease or on the isolation of *M. pulmonis* from joints (Harwick *et al.*, 1974). However, in contrast to our observations, these latter workers were only able to isolate *M. pulmonis* from a small percentage of arthritic joints from untreated mice (Harwick *et al.*, 1973*b*). The present study was therefore undertaken to re-examine the effect of tetracycline treatment on arthritic disease of mice inoculated several months previously with *M. pulmonis* and to correlate these observations with the recovery of mycoplasmas from joints.

MATERIALS AND METHODS

Mycoplasma medium and M. pulmonis.—The JB strain of *M. pulmonis* was the same as that described previously (Taylor, Taylor-Robinson and Slavin, 1974). Liquid medium for the isolation and growth of *M. pulmonis* consisted of Difco PPLO broth, fresh yeast extract, horse serum, glucose, phenol red, thallium acetate and penicillin G, in the proportions described previously (Taylor *et al.*, 1974). The same medium without thallium acetate was used to prepare a 3-day broth culture of *M. pulmonis* for inoculation of mice. It was dispensed in 1 ml aliquots and stored at −70°. On thawing, this stock culture contained 10^8 colour-changing units (ccu)/ml.

Mice.—Male C3H mice, weighing 20–30 g, were supplied by Scientific Agribusiness Consultants (International) Ltd. The specific-pathogen-free mice were produced to the M.R.C. Laboratory Animal Centre's Category 4 standard and were kept in isolation throughout the experimental period.

Induction and assessment of arthritis.—Mice were inoculated in the tail vein with 2×10^6 ccu of *M. pulmonis* in a volume of 0·2 ml. A subjective assessment of the severity of arthritis was made for each mouse by scoring the swelling of tarsal, carpal, metatarsal and metacarpal joints and digits from 1 to 3 as described previously (Taylor *et al.*, 1974). Scores for groups of mice were totalled, divided by the number of animals in each group and recorded as the "arthritis score".

Mycoplasma isolation.—Ankle joints were removed aseptically and dissected free from epidermis. The joints were cut in two and homogenized in a TenBröek grinder with mycoplasma medium to give a 10% (w/v) suspension. This was regarded as a 10^{-1} dilution of the original specimen and further ten-fold dilutions were made from it in myocplasma medium to determine the number of mycoplasmas. In some experiments one half of the ankle joint was examined for mycoplasmas and the other for histopathological changes. Isolated organisms were identified as *M. pulmonis* by the metabolism-inhibition test using hyperimmune rabbit serum (Taylor-Robinson *et al.*, 1966).

Histological procedures.—Joints for histological examination were fixed in 10% formol saline and then decalcified overnight in 10% formic acid. Sections of joints were stained with haematoxylin and eosin. The joint sections were coded and a subjective assessment of the severity of the inflammatory response in the joint tissues was made, by two independent observers, on a scale from 0 to 3. Dense infiltration was recorded as a score of 3. The scores of the two observers were in close agreement so they were averaged and recorded as a "lesion score".

Lymecycline treatment.—Lymecycline (Tetralysal, Carlo Erba, Milano) was diluted in sterile distilled water to give a suspension containing 30 mg lymecycline/ml. Mice were inoculated s.c. with approximately 0·2 ml of this suspension—*i.e.* 200 mg lymecycline/kg body wt—daily for a period of 16–19 days. Animals were examined for the presence of mycoplasmas in joints at 2, 3 or 24 days after treatment had been completed.

Determination of minimal inhibitory concentration (MIC) and mycoplasmacidal concentration (MCC) of lymecycline.—The MIC of lymecycline was determined in microtitre plates by a modification of the metabolism-inhibition test (Taylor-Robinson *et al.*, 1966). Serial dilutions of lymecycline were made in mycoplasma medium to give a range of concentrations from 0·000005 to 25 μg/ml. Approximately 10^4 ccu of *M. pulmonis* were added to each concentration of lymecycline. The MIC was regarded as the lowest concentration of lymecycline that prevented a change of 0·5 pH units in the mycoplasma medium after incubation at 37°. To determine the MCC of lymecycline the contents

of some of the microtitre wells not showing a pH change in the MIC test after 4 days' incubation were subcultured to 20 ml mycoplasma medium and further incubated. The lowest concentration of lymecycline from which mycoplasmas could not be re-isolated was regarded as the MCC.

To determine the level of lymecycline in the blood of mice after treatment, 6 mice were inoculated s.c. with 200 mg/kg body wt of lymecycline on 4 consecutive days, at the same time each day. Pairs of mice were exsanguinated 1, 16 and 24 h after the last injection and the blood from each pair was pooled, allowed to clot and the separated sera stored at −20°C. The MIC and MCC of the sera were determined as described above.

Zymosan-induced arthritis.—Mice were anaesthetized with ether. The knee joint was inoculated aseptically with 10 μl of a suspension containing 20 mg/ml of zymosan (Sigma Chemical Co., U.S.A.) in saline, by means of a 30-gauge needle attached to a Hamilton syringe. A sterile proliferative arthritis was induced in the inoculated joint as described previously (Keystone *et al.*, 1977).

RESULTS

Mycoplasmastatic and mycoplasmacidal activity of lymecycline

The MIC of lymecycline was 0·001 μg/ml. To determine the MCC of this compound, the contents of wells containing >3·0 μg/ml of lymecycline were subcultured and examined for mycoplasmas. *M. pulmonis* was not isolated from any of the samples that had been subcultured; thus the MCC of lymecycline of ⩽ 3·0 μg/ml.

The inhibitory levels of sera from lymecycline-treated mice, at various intervals after inoculation, are shown in Table I. Sera obtained from mice 1 h after lymecycline treatment were mycoplasmacidal at a dilution of at least 1/2.

TABLE I.—*Metabolism-inhibition Titre of Sera from Mice Previously Inoculated with Lymecycline*

Time after lymecycline treatment* (h)	M.I. titre of serum
1	256
16	128
24	32

* Mice inoculated s.c. with 6 mg lymecycline daily for 4 days.

Effect of lymecycline on M. pulmonis*-induced arthritis*

A preliminary experiment on small numbers of mice, inoculated 6 months and 1 to 1½ years previously with *M. pulmonis*, suggested that treatment with lymecycline had little effect on the severity of clinical arthritis. Thus there were no statistically significant differences in the arthritis score of lymecycline-treated mice and that of untreated, control animals regardless of the duration of the infection (Table II). However, there were significant differences in the recovery of mycoplasmas from the joints of lymecycline-treated and untreated mice ($P < 0{\cdot}01$, χ^2 test). Thus *M. pulmonis* was isolated from approximately 60% of arthritic joints from untreated mice, whereas mycoplasmas were not isolated from any clinically arthritic joints of lymecycline-treated animals (Table II). It is possible that the homogenized joints from lymecycline-treated mice contained sufficient antibiotic to inhibit multiplica-

TABLE II.—*Effect of Lymecycline on* M. pulmonis*-induced Arthritis of Mice*

		Untreated mice		Lymecycline-treated mice*	
Experiment No.	Days after infection	Arthritis score†	No. of mycoplasmas isolated from joints	Arthritis score	No. of mycoplasmas isolated from joints
1	168	2·8±0·5	—	2·3±0·6	—
	189	2·0±0·4	4·3±0·4‡ (7/12)*	1·4±0·3	0 (0/12)
2	365	2·3±0·7	—	2·2±0·5	—
	386	2·4±0·2	3·9±0·4 (8/13)	1·7±0·3	0 (0/11)

* Mice inoculated s.c. with 6 mg lymecycline daily for 19 days starting on Days 168 and 365 after infection.
† Mean of the amount of joint swelling ± s.d.
‡ Geometric mean, expressed as $\log_{10}$ ccu/ml ± s.d.
§ Proportion of clinically arthritic joints from which *M. pulmonis* was isolated.

tion of mycoplasmas *in vitro*. To examine this possibility, tubes containing 10^{-1} to 10^{-3} dilutions of joint homogenates from untreated mice, which failed to show a colour change after 12 days' incubation, and similar samples of joint homogenates from lymecycline-treated animals, were inoculated with 10^3 ccu of *M. pulmonis* and incubated at 37°. These tubes were examined at intervals for multiplication of *M. pulmonis*, as indicated by a change in the colour of the medium. All samples changed colour at approximately the same rate after inoculation with *M. pulmonis*.

Because of the small numbers of mice involved in this experiment and the possibility that the failure to isolate mycoplasmas from the joints of lymecycline-treated animals may have been due to residual antibiotic in the joint homogenate, the experiment was repeated using more animals, which were examined 3 and 24 days after termination of lymecycline treatment.

Eighty mice were inoculated i.v. with 2×10^6 ccu of *M. pulmonis* and examined for arthritis 5 months later. At this time, approximately 50% of mice still had arthritis which affected one or two joints of each animal. Mice with persistent joint inflammation were divided into two groups of about 20 mice with approximately equivalent arthritis scores, and one group was treated with lymecycline for 16 days, as described in Materials and Methods. The severity of arthritis in these two groups of mice was examined at various intervals throughout the course of lymecycline treatment. At necropsy, the ankle joints were examined microbiologically for the presence of mycoplasmas, and histologically for an inflammatory reaction. There were no significant differences in the clinical arthritis scores of the two groups of mice 3 and 24 days after termination of lymecycline treatment (Table III). However, as seen in Table IV, the severity of the inflammatory reaction, as indicated by the lesion score, in the ankle joints of mice 3 and 24 days after lymecycline treatment was significantly less than that of the control animals ($P < 0{\cdot}001$ and $P < 0{\cdot}002$ respectively; Student's t test). Periarticular tissues of clinically arthritic joints from untreated mice were densely infiltrated with polymorphonuclear (PMN) leucocytes and mononuclear cells (Fig. 1a and b). In addition, the synovial membrane was hyperplastic and there was an exudative inflammatory reaction in the joint space (Fig. 1c). In contrast, periarticular tissues of clinically arthritic joints from

TABLE III.—*Effect of Lymecycline on the Arthritis Score of Mice Inoculated I.V. 20 Weeks Previously with* M. pulmonis

Group No.	Days after infection	Arthritis score of: Untreated mice	Lymecycline-treated mice*
1	140	$2{\cdot}1 \pm 1{\cdot}8$†	$1{\cdot}8 \pm 0{\cdot}8$
	159‡	$2{\cdot}5 \pm 2{\cdot}2$	$1{\cdot}4 \pm 0{\cdot}8$
2	140	$2{\cdot}4 \pm 1{\cdot}9$	$2{\cdot}7 \pm 1{\cdot}8$
	180§	$2{\cdot}7 \pm 2{\cdot}2$	$2{\cdot}5 \pm 1{\cdot}5$

* Mice inoculated s.c. with 6 mg lymecycline daily for 16 days starting 140 days after infection.
† Mean of the amount of joint swelling ± s.d.
‡ 3 days after termination of lymecycline treatment.
§ 24 days after termination of lymecycline treatment.

TABLE IV.—*Effect of Lymecycline on Arthritic Disease of Mice Inoculated I.V. 20 Weeks Previously with* M. pulmonis

Days after infection	Untreated mice: Lesion score†	Untreated mice: No. of mycoplasmas isolated from joints	Lymecycline-treated mice*: Lesion score	Lymecycline-treated mice*: No. of mycoplasmas isolated from joints
159	$1{\cdot}5 \pm 0{\cdot}6$	$3{\cdot}6 \pm 0{\cdot}4$‡ (8/10)§	$0{\cdot}4 \pm 0{\cdot}2$	0 (0/7)
180	$1{\cdot}1 \pm 0{\cdot}5$	$4{\cdot}7 \pm 1{\cdot}2$ (10/10)	$0{\cdot}4 \pm 0{\cdot}3$	0 (0/10)

* Mice inoculated s.c. with 6 mg lymecycline daily for 16 days starting 140 days after infection.
† Mean amount of cellular infiltration in joints ± s.d.
‡ Geometric mean, expressed as $\log_{10}$ ccu/ml ± s.d.
§ No. of joints from which mycoplasmas were isolated/no. of joints examined with cellular infiltration.

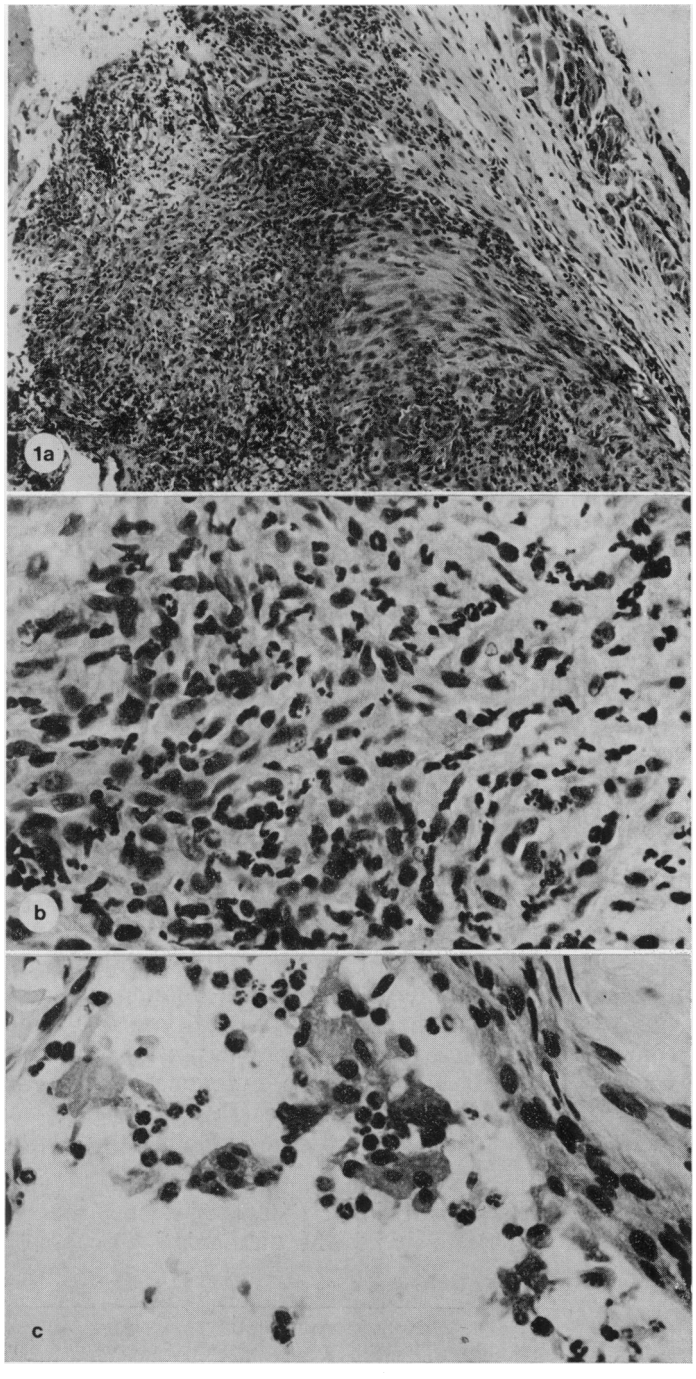

FIG. 1.—Photomicrographs of joints from mice inoculated i.v. 5 months previously with *M. pulmonis*. (a) Dense cellular infiltration of periarticular tissues (×115). (b) Cellular infiltration is composed of PMN leucocytes and mononuclear cells (×475). (c) Exudative inflammatory reaction in joint space (×475).

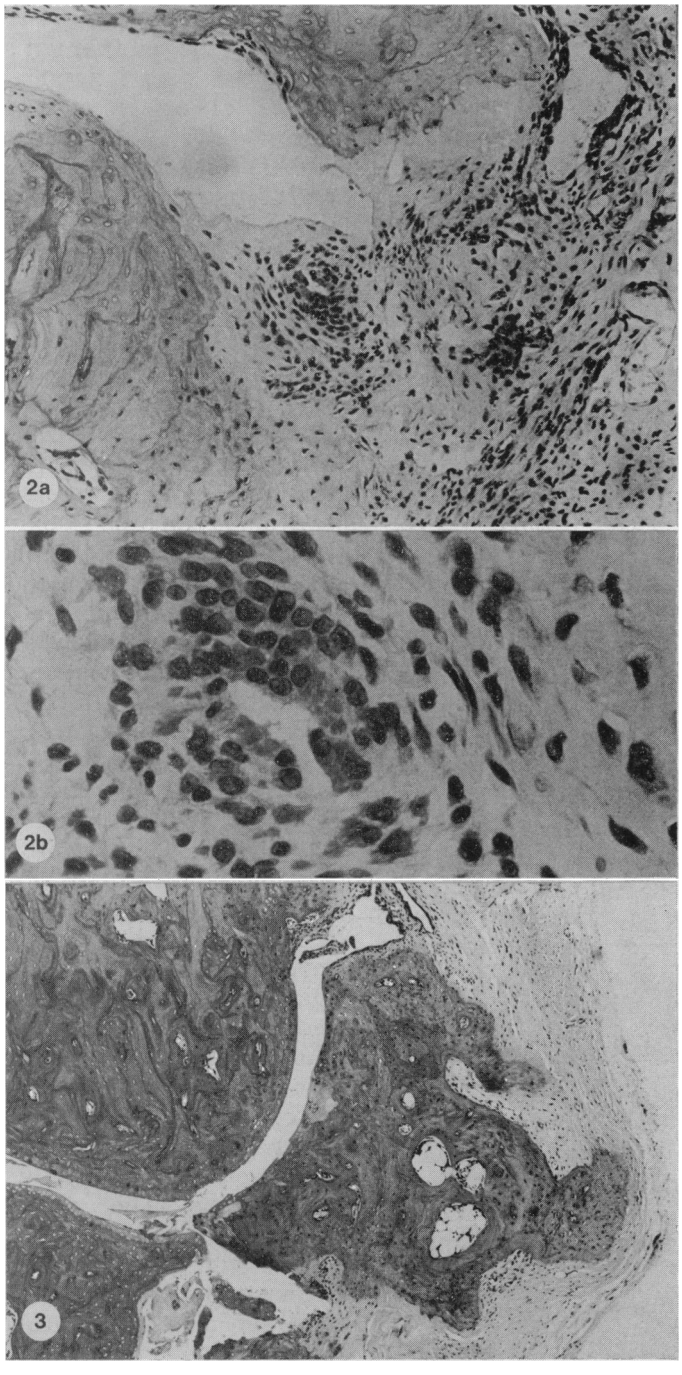

FIG. 2.—Photomicrographs of joints from mice inoculated i.v. 5 months previously with *M. pulmonis*, 3 days after lymecycline treatment had ended. (a) Slight cellular infiltration of periarticular tissues (× 115). (b) Cellular infiltrate is composed of mononuclear cells (× 475).

FIG. 3.—Photomicrograph of joint from mouse inoculated i.v. 5 months previously with *M. pulmonis*, 24 days after lymecycline treatment had ended. Very little inflammation (× 45).

mice examined 3 days after lymecycline treatment were only slightly infiltrated with cells (Fig. 2a) which were mainly mononuclear (Fig. 2b). Furthermore, there was little or no inflammatory reaction in the joint spaces. Twenty-four days after lymecycline treatment there were only minimal infiltrations of mononuclear cells in periarticular tissues (Fig. 3).

As observed previously, *M. pulmonis* was not isolated from any of the clinically arthritic joints of lymecycline-treated mice examined 3 or 24 days after treatment had ended. In contrast, mycoplasmas were isolated from nearly all joints from untreated animals which showed an inflammatory response histologically (Table IV).

Effect of lymecycline on zymosan-induced arthritis

To determine whether the antibiotic had any direct effect on the inflammatory response, the severity of zymosan-induced arthritis in lymecycline-treated mice was compared with that in untreated animals. Mice were inoculated s.c. with 200 mg lymecycline/kg body wt for 7 days before and for 3 days after intra-articular injection with zymosan. Animals were killed 3 and 10 days after inoculation with zymosan and the knee joints examined histologically. Three days after inoculation with zymosan, periarticular tissues of knee joints from control animals were infiltrated with PMN leucocytes. This infiltrate was replaced by mononuclear cells 10 days after inoculation. There were no significant differences in the severity or cellular nature of the inflammatory reaction of control and lymecycline-treated mice examined 3 or 10 days after inoculation with zymosan.

DISCUSSION

Preliminary investigations indicated that mycoplasmas were isolated from only 60% of clinically arthritic joints of mice inoculated 6 to 18 months previously with *M. pulmonis*. In other studies, histological examination of clinically arthritic joints at this time showed that not all of them were infiltrated with inflammatory cells. The clinical abnormalities in such joints appeared to be the result of periarticular fibrosis with resultant ankylosis (Taylor and Taylor-Robinson, unpublished observations). Thus the clinical assessment of arthritic joints may not accurately reflect the degree of articular inflammation, especially during the chronic phase. Further, it was possible that mycoplasmas were associated mainly with joints which were undergoing an inflammatory reaction, and were not present when the inflammatory response had subsided. To investigate this further, joints were cut in two so that each joint could be examined both microbiologically and histopathologically. This procedure, which was most readily performed on ankle joints, showed that there was a good correlation between the isolation of mycoplasmas and the severity of cellular infiltration in the joints (see Table IV). These findings were in contrast to those of Harwick *et al.* (1973*a*), who isolated *M. pulmonis* from only 27% of clinically arthritic joints at all stages of the disease. This low isolation rate may have been due in part to resolution of the cellular inflammatory reaction in some of the clinically arthritic joints examined for mycoplasmas. Further, it may be that mice of different strains vary in their response to *M. pulmonis* infection. Thus chronic arthritis may occur in some mouse strains because of the persistence of viable mycoplasmas in the joints, whereas in other strains chronic arthritis may be the result of other, possibly immunological mechanisms. This possibility is being investigated further.

The failure of lymecycline treatment to reduce the severity of clinical arthritis in mice inoculated 5 months or more previously with *M. pulmonis* is in agreement with the observations of Harwick *et al.* (1974). However, in contrast to these workers, we found that the recovery of mycoplasmas from clinically arthritic joints of lymecycline-treated mice was

significantly different from that of untreated controls. Our failure to isolate mycoplasmas from the joints of treated mice could have been due to residual antibiotic in the joint homogenate. Although this does not seem likely, in order to reduce the possibility in subsequent experiments, joints were examined 3 and 24 days after lymecycline treatment had ended, at which times antibiotic levels in the blood and tissues should be minimal. Even under these circumstances, *M. pulmonis* organisms were not isolated from any joints of lymecycline-treated animals, whereas they were recovered from 80–100% of the arthritic joints of untreated mice.

The failure of Harwick *et al.* (1974) to eliminate *M. pulmonis* from chronically arthritic joints by treating mice with rolitetracycline may have been due to the antibiotic failing to penetrate into the joints or being present in insufficient concentration. The ways in which lymecycline cleared the mycoplasmas from the joints are not clear. Since this antibiotic reaches cidal levels in the sera of mice 1 h after s.c. inoculation, it may act by killing the organisms directly if similar levels are attained in the joints. Alternatively, if the amount of antibiotic present in the joint is mycoplasmastatic, then the host's defence mechanisms may be able to clear the organisms.

The clearance of mycoplasmas from the joints was associated with a marked decrease in the numbers of PMN leucocytes and mononuclear cells in the periarticular tissues. This suggests that the stimulus for the persistent inflammatory reaction in the joints was the continued presence of viable organisms. The reasons for the persistence of organisms are not clear. It is known that sera from mice inoculated i.v. 6 weeks previously with *M. pulmonis* can confer on recipient animals resistance to *M. pulmonis*-induced arthritis (Taylor and Taylor-Robinson, 1977). It may be, however, that protective antibodies in the sera do not gain sufficient access to the joints to clear the organisms. Thus, an equilibrium may exist between replication of the mycoplasma and inactivation by the host, resulting in its persistence in the joint. When this equilibrium is disturbed as a result of lymecycline treatment, the mycoplasmas are cleared and the stimulus for the inflammatory reaction disappears.

It is possible that the amount of lymecycline used in this investigation was cytotoxic. However, the failure of the antibiotic to effect the severity of zymosan-induced arthritis, which is microbiologically sterile, indicates that the beneficial effect of lymecycline was not due to a direct immunosuppressive effect on the inflammatory cells. Thus these studies indicate that persistence of viable mycoplasmas is necessary for the chronicity of *M. pulmonis*-induced arthritis in C3H mice.

As mentioned previously, tetracycline is most effective in reducing the severity of arthritis when treatment is started at the time of mycoplasma infection (Hannan, 1977; Harwick *et al.*, 1974). The failure to eradicate mycoplasmas from an established infection is a common feature of many mycoplasma-induced diseases (Arisoy *et al.*, 1967; Fabricant, 1969). For example, treatment of *M. pneumoniae* infection in man with tetracycline produced some remission of symptoms but had little effect on the excretion of organisms from the respiratory tract (Shames *et al.*, 1970; Smith, Friedewald and Chanock, 1967). It is perhaps surprising, therefore, that lymecycline treatment of chronically arthritic mice not only reduced the severity of the inflammatory reaction in the joint, but also cleared the mycoplasmas from this site. Intensive therapy with this compound may be useful in the treatment of other mycoplasma infections.

The failure of tetracycline-treatment to alter the course of rheumatoid arthritis has been interpreted as meaning that mycoplasmas are not involved in the pathogenesis of this disease (Skinner *et al.*, 1971). However, this is not an entirely acceptable argument in view of the difficulty of eradicating organisms from known myco-

plasma infections with tetracyclines. A double-blind trial with a compound such as lymecycline, known to be effective in the treatment of a chronic mycoplasma infection, should perhaps be carried out, before dismissing persistence of viable mycoplasmas as a factor in the pathogenesis of rheumatoid arthritis.

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